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(54) Title: ICAM-1 SELECTIVE ECHOGENIC MICROBUBBLES

(57) Abstract

Methods of preparing gas-filled or gas precursor-filled microbubbles with attached ICAM-binding molecules are described. Gas-filled microbubbles conjugated to anti-ICAM antibodies or other ICAM-binding molecules are useful, for example, in ultrasonic imaging of vascular endothelial cell dysfunction, and in therapeutic-drug-delivery-or-gene therapy-directed to dysfunctioning endothelial cells.

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ICAM-1 SELECTIVE ECHOGENIC MICROBUBBLES

FIELD OF THE INVENTION

This invention has applications in the fields of medical imaging technology and targeted drug delivery. The invention relates to echogenic contrast agents for ultrasound imaging of body tissues and organs. The contrast agents of the invention are gas-filled microbubbles, which are prepared by methods known in the fields of liposome and microsphere synthesis. The microbubbles of the invention are conjugated to ICAM-1 binding molecules, such as anti-ICAM-1 antibodies, which direct the binding of the microspheres to ICAM-1 expressing cells, thereby rendering such cells visible in a sonogram.

The microbubbles of the invention are also expected to have utility as drug delivery vehicles. The microbubbles are capable of concentrating an encapsulated therapeutic agent at ICAM-1 expressing cells and, are capable of releasing the therapeutic agent upon application of ultrasound radiation.

BACKGROUND OF THE INVENTION

1. Medical imaging technology.

A variety of imaging techniques have been used to detect and diagnose disease in animals and humans. X-ray imaging is one of the first techniques used for diagnostic imaging. The images obtained through this technique reflect the electron density of the object being imaged. Contrast agents such as barium or iodine have been used over the years to attenuate or block X-rays such that the contrast between various structures is increased. X-rays, however, are known to be somewhat dangerous, since the radiation employed in X-rays is ionizing, and the various deleterious effects of ionizing radiation are cumulative. Also, X-ray imaging is not well suited to the detection of subtle differences among soft tissues. With respect to imaging the vasculature, contrast agents make it possible to visualize the gross anatomy of a blood vessel, and can reveal blockage or dilation, but X-ray technology cannot detect biochemical or cellular aberrations.

A more recent technique is magnetic resonance imaging (MRI). This technique has some drawbacks, such as high capital and operating expenses, and the fact that it cannot be conducted as a portable examination. MRI facilities are not widely available, and tend to be located at major hospitals and medical centers. MRI in general has a low resolution in time, and therefore is not well suited for imaging structures that are in motion, such as the heart, lungs, and large vasculature.

Radionuclides, employed in nuclear medicine, provide another imaging technique. In employing this technique, radionuclides such as technetium labeled compounds are injected into the patient, and images are obtained from gamma cameras. Nuclear medicine techniques suffer from poor spatial resolution and expose the patient to the deleterious effects of radiation. Furthermore, the production, handling and disposal of radionuclides are problematic. The spatial resolution is not adequate for useful vascular imaging.

Ultrasound is a diagnostic imaging technique which, unlike nuclear medicine and X-rays, does not expose the patient to the harmful effects of ionizing radiation. Moreover, unlike magnetic resonance imaging, ultrasound is relatively inexpensive and can be conducted in a physician's office, or in a hospital room as a portable examination. In the ultrasound technique, sound is transmitted into the patient via a transducer. The sound waves propagate through the body, and are reflected or absorbed to varying degrees depending on the acoustic properties of the tissues and fluids in the body. Interfaces between regions of differing acoustic impedance are particularly reflective. Sound waves reflected by interfaces are detected by a receiver in the transducer and processed to form an image. In Doppler mode, ultrasonic imaging can also detect differences in direction of blood flow.

Advances have been made in recent years in ultrasound technology. However, despite these various technological improvements, ultrasound is still an imperfect tool in a number of respects, particularly with regard to the imaging and detection of disease in the liver and spleen, kidneys, heart and vasculature, and with regard to measuring blood flow. The ability to detect, image, and measure in these regions

depends on the difference in acoustic properties between tissues or fluids and the surrounding tissues or fluids, and such differences are often slight. As a result, contrast agents have been sought which will increase the acoustic difference between the tissues or fluids of interest and the surrounding tissues or fluids, in order to improve ultrasonic imaging and disease detection.

The principles underlying image formation in ultrasound have directed researchers to the pursuit of gaseous contrast agents. Changes in acoustic properties or acoustic impedance are most pronounced at interfaces of substances with greatly differing density or acoustic impedance, particularly at interfaces between solids, liquids and gases. When ultrasound waves encounter such interfaces, the change in acoustic impedance results in an intense reflection of sound waves and an intense signal in the ultrasound image. Another factor affecting the efficiency or reflection of sound is the elasticity of the reflecting interface. The greater the elasticity of this interface, the more efficient the reflection of sound. Substances such as gas bubbles present highly elastic interfaces, consequently researchers have focused on the development of ultrasound contrast agents based on gas bubbles or gas-containing materials, and on efficient methods for their preparation.

Despite these advances, ultrasound imaging, like X-ray angiography, can only reveal the gross anatomy of the vasculature, and changes in gross anatomy usually do not appear until the underlying disease has progressed to the point where significant damage has taken place. There remains a need for a method of imaging changes at the biochemical or cellular level, particularly those changes associated with the early stages of vascular disease, in order to enable the early detection of vascular disease before substantial damage has occurred. Early diagnosis and therapeutic intervention could avoid the expensive surgical intervention that is often necessary to deal with damaged vasculature.

2. Targeted drug delivery.

Another area of significant research effort is in the area of targeted drug delivery. Targeted delivery means are particularly important where toxicity is an

issue. Specific therapeutic delivery methods potentially serve to minimize toxic side effects, lower the required dosage amounts, and decrease costs.

Better means of delivery for therapeutics such as genetic materials and toxic drugs are needed to treat a wide variety of human and animal diseases. Great strides have been made in characterizing genetic diseases and in understanding protein transcription but relatively little progress has been made in delivering genetic material to cells for treatment of human and animal disease.

The methods and materials in the prior art for introduction of genetic materials, for example, to living cells are limited and ineffective. To date, several different methods have been developed. These methods include techniques such as calcium phosphate precipitation and electroporation, carriers such as cationic polymers and aqueous-filled liposomes, and adenoviral vectors. These methods have all been relatively ineffective *in vivo* and are primarily of use for cell culture transfection. None of these methods enable targeted local delivery, release, and integration of genetic material to the target cell.

A principal difficulty has been to deliver the genetic material from the extracellular space to the intracellular space. Even more difficult is the task of effectively localizing genetic material at the surface of the targeted cell membranes. A variety of techniques have been tried *in vivo* but without great success. For example, viruses such as adenoviruses and retroviruses have been used as vectors to transfer genetic material to cells. Whole virus has been used but the amount of genetic material that can be placed inside of the viral capsule is limited, and there is concern about the hazards of employing an infectious, live virus. The essential components of the viral capsule may be isolated and used to carry genetic material to selected cells. *In vivo*, however, not only must the delivery vehicle recognize certain cells but it also must deliver its contents to these cells. Despite extensive work on viral vectors, it has proven difficult to develop a successfully targeted viral vector for delivery of genetic material *in vivo*.

Conventional liquid-containing liposomes have been used to deliver genetic

material to cells in cell culture but have generally been ineffective *in vivo* for cellular delivery of genetic material. For example, cationic liposome transfection techniques have not worked effectively. More effective means are needed to improve the cellular delivery of therapeutics such as genetic material, and a need exists for methods for targeting and concentrating liposomes at the surface of the target cells.

3. Diagnosis of myocardial disease.

The development of myocardial ischemia is more complex than the previously held, simplistic view of progressive stenosis and blood supply limitation. It has become increasingly clear that a derangement of the normal functional status of the vascular endothelium plays a prominent role in the early biology of atherosclerosis. When endothelial cells are injured or activated, the endothelial surface becomes prothrombotic and leukocyte adhesive, and loses its ability to vasodilate. However, these changes in functional status cannot be assessed by current methods of angiography. Consequently, the angiographic extent of coronary disease cannot fully predict cardiac outcome in patients.

Atherogenesis appears closely linked to inflammation, and it is likely that the earliest detectable lesions, which predate clinical disease, involve monocyte adhesion to the endothelium. It is probable that the latter is mediated by up-regulation of specific leukocyte adhesion molecules (LAMS). For example, a monocyte LAM locally expressed by endothelium overlying early foam-cell-lesions has been identified in hyperlipidemic rabbits. Others have demonstrated the presence of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) in atherosclerotic plaques. Hypercholesterolemia is associated with endothelial dysfunction in humans, and with macrophage recruitment, dysfunction of regenerated endothelium, and rapid induction of VCAM-1 in rabbits, suggesting a relationship between endothelial dysfunction and expression of LAMS.

Despite the growing body of experimental and clinical evidence implicating the role of endothelial dysfunction in the development of ischemic heart disease, current methods for identifying endothelial dysfunction in the clinical setting are

limited. The potential therapeutic opportunities afforded by the detection of endothelial dysfunction in humans have not yet been realized. Current in-vivo approaches to evaluating endothelial function include invasive techniques in the cardiac catheterization laboratory which do not completely assess the endothelium at the microvascular level. Non-invasive methods employing echocardiographic estimates of brachial artery reactivity, which are extrapolated to coronary endothelial function, are indirect and limited in resolution.

Recently, it has been reported that albumin microbubbles, which are used in myocardial contrast echography (MCE), tend to linger in the myocardium where there is a pathophysiological state associated with endothelial dysfunction. The mechanism behind this phenomenon is unknown, but experiments have shown that albumin microbubbles adhere in small but significant numbers to activated, or inflamed, human coronary endothelial cells in culture, but not to normal or resting endothelial cells (Villanueva *et al.*, 1997, *J. Am. Coll. Cardiol.*, **30**, 689-693, and references therein).

4. Microbubble and liposome technology.

Ryan *et al.*, in U.S. patent 4,544,545, report phospholipid liposomes having a chemically modified cholesterol anchored in the bilayer. An aqueous medium, containing a tracer, therapeutic, or cytotoxic agent, is confined within the liposome.

D'Arrigo, in U.S. Patents 4,684,479 and 5,215,680, teaches a gas-in-liquid emulsion and method for the production thereof from surfactant mixtures. The '479 patent reports the production of liposomes by shaking a solution of the surfactant in a liquid medium in air. The '680 patent is directed to a large-scale method of producing lipid coated microbubbles.

Ilum and Johnson, in U.S. Patent 5,648,095 report a method for making gas-filled microbubbles, which involves preparing volatile oil-filled microcapsules and then evaporating the volatile oil to leave hollow microspheres.

WO 80/02365 reports the production of microbubbles having an inert gas, such as nitrogen; or carbon dioxide, encapsulated in a gellable membrane.

Sutton *et al.*, in European Patent application publication number EP 0681843 A2, report a spray-drying method for preparing hollow microbubbles of albumin or gelatin.

WO 82/01642 describes microbubble precursors and methods for their production.

Feinstein, in U.S. Patents 4,718,433 and 4,774,958, reports the use of albumin coated microbubbles for the purposes of ultrasound imaging.

Widder, in U.S. Patents 4,572,203 and 4,844,882, reports a method of ultrasonic imaging and a microbubble-type ultrasonic imaging agent.

Quay *et al.*, in European Patent application, publication number EP 0727225 A2, reports a method of preparing gas-filled microbubbles with CAM ligands attached. The ligands (small peptides and carbohydrates) are conjugated to fluorinated surfactants, and the conjugates are combined with the gas precursor perfluoropentane and a surfactant and agitated to produce an emulsion of perfluoropentane in water. The ability of the resulting microbubbles to bind to CAM was not demonstrated. Anti-CAM antibodies were mentioned as potential CAM ligands, but no example of antibody conjugation was disclosed.

Quay, in WO 93/05819 and in related U.S. Patent 5,558,853, reports the use of gas precursors to form microbubbles comprising certain gases, which are selected based upon their known physical constants. The gas precursors are provided as liquid-in-liquid emulsions, which are converted into the gas phase at body temperature.

Schneider *et al.*, in European Patent specification, publication number EP 0554213 B1, report the loading of microbubbles with similarly selected gases, the selection being based on the "critical pressure" of the gases. The critical pressure is dependent on numerous factors, and appears to be related to the solubility, molecular weight, and concentration of the gas.

Lanza *et al.*, in WO 93/20802, reports a class of multi-lamellar liposomes, which although not gas-filled are substantially echogenic. Conjugation of an IgG

antibody to the multilamellar liposomes is also reported. This group has published similar information: Alkan-Onyuksel *et al.*, *J. Pharm. Sci.*, 85, 486-490 (1996).

Demos *et al.*, in *J. Pharm. Sci.*, 86, 167-171 (1997), reported the conjugation of multi-lamellar liposomes to anti-fibrinogen antibodies, and the use of these targeted liposomes to image thrombi *in vitro*. Anti-ICAM-1 antibodies were also conjugated to the liposomes, but these liposomes were reported only in control experiments, where they were observed not to bind to fibrin-coated disks.

Demos *et al.*, in *Circulation*, 94 (8 Suppl.), I209 (1996), and in *J. Investig. Med.*, 44, 392A (1996), reported that liposomes conjugated to anti-ICAM-1 antibodies attached to early-stage atherosclerotic plaques. These liposomes, however, were not gas-filled.

Lanza and Wickline, in International publication WO 96/41647, report a method of targeting liposomes to selected epitopes by means of biotin-avidin interactions. A biotinylated antibody to the epitope is allowed to bind to the epitope, avidin is then applied, and finally a biotinylated liposome is allowed to bind to the avidin. Antibody-conjugated liposomes are not independently prepared in this method.

Kaufman *et al.*, in U.S. Patent 5,171,755, report an emulsion comprising a highly fluorinated organic compound, an oil having no substantial surface activity or water solubility and a surfactant. Kaufman *et al.* also report a method of using the emulsion in medical applications.

Bernstein *et al.*, in U.S. Patent 5,611,344, report microbubbles filled with fluorinated gases, which are stated to have improved echogenic and stability properties. Bernstein *et al.* report the incorporation of bioadhesive polymers, particularly the polylactide/polyglycolide/poly(ethylene glycol) copolymer, into the microbubbles, for purposes of imaging mucosal surfaces. Bernstein *et al.* also mention that the microbubbles can be targeted by attachment of ligands, including antibodies, but neither examples nor references to such attachment are provided.

Tournier *et al.*, in European Patent application EP 0638318 A2, describe

liposomes incorporating bioadhesive polymers similar to those of Bernstein *et al.*, with a similar affinity for mucosal surfaces. Polylactic acid and polyacrylate/polyacrylic acid copolymers are employed by Tournier *et al.*

Lohrmann, in U.S. Patent 5,562,893, reports gas-filled microspheres which are prepared from fluorine-containing amphiphilic materials. These microspheres exhibit improved stability with respect to loss of the encapsulated gas.

Klibanov *et al., Acta Radiologica* , 38, 113-120 (1997), demonstrated the utility of adherent gas-filled microbubbles for ultrasound imaging, at surface densities less than 0.8%, using conventional ultrasound systems.

McCreery *et al., Circulation*, 96, I-213 (1997) recently developed a microbubble that binds directly to thrombus.

A need exists for a non-invasive method for directly assessing coronary endothelial function at the cellular level. Despite the considerable amount of work by others in the field, it has not heretofore been possible to image by ultrasound activated endothelial cells in intact tissue. There remains a need for such an imaging method, in order to detect at an early stage the precursor cellular events that lead to vascular disease and coronary damage.

SUMMARY OF THE INVENTION

Prior to the present invention, it had not been demonstrated that one could directly visualize the specific adherence of gas-filled, targeted microbubbles to the surface of activated coronary endothelial cells *in situ*. The present invention provides gas-filled microbubbles conjugated to at least one molecule having binding specificity for an antigen associated with inflamed or activated endothelial cells. An example of such an antigen is ICAM-1. Gas-filled microbubbles conjugated to an anti-ICAM-1 antibody, prepared and used according to the present invention, permit for the first time the visualization of microbubbles adherent to activated coronary endothelial cells *in situ*.

In one embodiment of the invention, the molecules having affinity for ICAM-1 are antibodies, which serve to target the microbubbles to ICAM-1-expressing

endothelial cells, which are associated with the early stages of atherosclerosis. The microbubbles of the invention, once bound to the ICAM-1 expressing endothelial cells, serve as echogenic ultrasound contrast agents and are expected to enable for the first time convenient visualization of damaged vascular endothelium, before any angiographic evidence would be noticeable. The targeted microbubbles of the invention also serve as drug delivery vehicles, particularly for genetic therapy by delivery of nucleic acids, for damaged vascular endothelial cells.

Accordingly, the present invention provides gas-filled microbubbles conjugated to a molecule having binding affinity for ICAM-1. In a preferred embodiment, the molecule having binding affinity for ICAM-1 is an anti-ICAM-1 antibody, due to the ready availability of such antibodies. The preferred size of the microbubbles is between 0.1 and 10 microns, and more preferably between 1 and 5 microns. The preferred method of attachment of the antibody to the microbubbles is via a flexible linker, most preferably a poly(ethylene glycol) linker.

The invention also provides an echogenic contrast agent preparation, comprising a suspension of the conjugated microbubbles of the invention in a pharmaceutically acceptable vehicle. Suitable vehicles include sterile water and sterile normal saline, with optional additives such as stabilizers and buffers.

The invention also provides a method for imaging a tissue, or a portion of the tissue, which comprises ICAM-1 expressing cells. The method comprises the steps of:

a. contacting the tissue with gas-filled microbubbles conjugated to a molecule having binding affinity for ICAM-1,

b. allowing the microbubbles to bind to the cells, and

c. obtaining an ultrasound image of the tissue.

In a preferred embodiment of the above method, the molecule having binding affinity for ICAM-1 is an anti-ICAM-1 antibody.

The method is particularly applicable to the imaging of activated coronary endothelial cells, most particularly to such cells located in the coronary vasculature.

This invention also provides methods for preparing and using gas-filled or gas-

precursor-filled liposomes conjugated to molecules having affinity for ICAM-1, such as anti-ICAM-1 antibodies. Unlike the methods of the prior art which are directed to the formation of antibody-conjugated liposomes with an aqueous solution filling the interior, the methods of this invention are directed to the preparation and use of antibody-conjugated gas-filled microbubbles, which are far more echogenic.

It is an object of this invention to provide a method employing conjugated gas-filled microbubbles together with myocardial contrast echography to study specific cellular features of coronary endothelium in real-time. It is also an object of this invention to provide a non-invasive method for directly assessing coronary endothelial function at the cellular level, as an aid to the early diagnosis of coronary disease, particularly atherosclerosis.

It is a further object of this invention to provide a new ultrasound contrast agent, comprising gas-filled microbubbles conjugated to an molecule having binding affinity for ICAM-1. The molecule is preferably an antibody specific for an antigen which is expressed by activated or inflamed coronary endothelial cells. The antibody is preferably an antibody to an intracellular adhesion molecule (ICAM), and is more preferably an antibody to human ICAM-1. The targeted microbubbles of this invention bind specifically to inflamed or activated human coronary artery endothelial cells over-expressing ICAM-1. These targeted microbubbles are expected to enable the in vivo echocardiographic detection of expressed ICAM-1, a marker associated with endothelial dysfunction, and hence enable the non-invasive detection of atherosclerosis in its earliest, pre-clinical stages. It is expected that the method of this invention will also enable early prediction of the risk of restenosis after an angioplasty procedure.

The targeted microbubbles of this invention are also expected to be useful for the effective targeted delivery of therapeutics to inflamed vascular endothelial cells; accordingly it is a further object of this invention to provide microbubbles conjugated to anti-ICAM-1 antibodies which carry therapeutic agents, and which can release or be induced to release these therapeutic agents in the vicinity of inflamed or activated

endothelial cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Adherence of the 3 microbubbles species to normal vs. activated ECs. Adherence increased when inflamed ECs were exposed to microbubbles containing anti-ICAM-1.

Figures 2A-E: Fluorescent micrographs of ECs after exposure to either non-specific IgG-conjugated bubbles (2A and 2B) or anti-ICAM-1-conjugated bubbles (2C and 2D). Figure 2E is an enlargement of a single activated cell exposed to anti-ICAM-1 bubbles.

Figure 3: Flow cytometry indicating the relative distribution of ICAM-1 binding (number of events vs. fluorescent intensity) in unstimulated (B) and IL1-β stimulated (C) ECs. Background non-specific IgG binding is shown in (A).

DETAILED DESCRIPTION OF THE INVENTION

The term "microbubble" as used herein designates hollow spheres or globules, of a size between about 10 nanometers and 100 microns, filled with air, gas, or a gas mixture, and having a material boundary, envelope, or wall. The wall may comprise lipids, in particular lipid monolayers, or may be composed of other materials known to the art such as synthetic polymers, proteins, polysaccharides, and other biopolymers, amphiphilic agents, and the like. Liposomes, whether monolamellar or polylamellar, fall under the microbubble designation when they contain a gas in their interior space. Liposomes or other microscopic hollow spheres, when filled with a gas precursor, will be understood to be equivalent to gas-filled microbubbles for the purposes of this invention.

As used herein, the term "gas precursor" denotes a compound which, at a selected activation or transition temperature, changes phases from a liquid to a gas. The gaseous precursor may be selected so as to form the gas in situ in the targeted tissue or fluid, in vivo upon entering the patient or animal, prior to use, during storage, or during manufacture. Methods of producing the gas precursor-filled microspheres

are known in the art, for example as detailed in U.S. Patent 5,469,854, the contents of which are incorporated herein by reference.

The term "molecule having binding affinity for ICAM-1" refers to a molecule which binds selectively to ICAM-1 in the presence of other extracellular proteins and membranes. Examples include, but are not limited to, anti-ICAM-1 antibodies, the beta-2 integrin subunit, alphaL/beta2 integrin (LFA-1), alphaM/beta2 integrin (Mac-1), and the like. Integrin subunits required for preparation of ICAM-1-binding integrins are available by recombinant techniques (Venstrom and Reichardt, *Mol. Biol. Cell*, **6**, 419-431 (1995)). Lee *et al., Virus Genes* **9**, 177-181. Rhinovirus coat proteins are known to bind specifically to ICAM-1, and it is anticipated that these proteins might also be used in the present invention (Olson *et al., PNAS*, **90**, 507-511 (1993)). Synthetic peptides and polypeptides having selective binding affinity for ICAM-1, which may be obtained by known methods, (Ladner *et al.*, US patent 5,223,409; Kay *et al.*, US patent 5,498,538), may also be employed in the present invention. The binding affinity of such molecules for ICAM-1 may be readily determined by known methods, such as for example that of Casasnovas and Springer, *J. Biol. Chem.*, **270**, 13216-13224 (1995).

The methods of this invention provide, among other things, for agitating an aqueous solution comprising a lipid in the presence of a gaseous precursor. The concentrations of lipids will in general be those known to be appropriate for liposome formation. In one embodiment, the concentration of 1,2-dipalimitoyl phosphatidylcholine (DPPC) used to form gas precursor-filled liposomes according to the methods of this invention is about 20 mg/ml to about 30 mg/ml saline solution. The concentration of distearoyl phosphatidylcholine (DSPC) used in preferred embodiments is about 5 mg/ml to about 10 mg/ml saline solution.

According to the methods of this invention, the local ambient atmosphere may also provide the gas. The local ambient atmosphere may be the atmosphere within a sealed container, or in an unsealed container, may be the external environment. Alternatively, a gas may be injected into or otherwise added to the container having

the aqueous lipid solution or into the aqueous lipid solution itself in order to provide a gas other than air. Preferred gases are perfluorinated hydrocarbons, more preferably those with five or fewer carbons, most preferably perfluoropentane or a perfluorobutane. Accordingly, this invention includes entrapment of air and/or other gases along with or instead of gas precursors.

Since perfluorocarbon gases are less soluble and diffusable than most other gases, they tend to be more stable when entrapped in microbubbles, even when the microbubbles are composed of lipids in the liquid-crystalline state, such as liposomes composed of phosphatidylcholine. In this case, techniques such as microemulsification are preferred for forming the liposomes which entrap the gas precursor. A microfluidizer (Microfluidics, Newton, Mass.) is particularly useful for making an emulsion of small liposomes which entrap a gas precursor.

Where the gas or gas precursor-filled microbubbles are to contain a therapeutic compound, the therapeutic compound may be combined together with the microbubble precursors before the gas or gas precursor is entrapped. Alternatively, the therapeutic compound may be added after the microbubble formation process, whereupon the microbubbles are coated on the outside with a therapeutic compound.

Microbubbles of this invention include but are not limited to liposomes, and may comprise lipids, steroids, proteins, polysaccharides, polyethers, and optionally cross-linked polymers. The liposomes may be formed as monolayers or bilayers and may or may not have a stabilizing coating.

Lipids bearing hydrophilic polymers such as poly(ethylene glycol) (PEG), including and not limited to PEG 2,000 MW, 5,000 MW, and PEG 8,000 MW, are useful for improving the stability and size distribution of the gas or gas precursor-containing microbubbles. Various different mole ratios of PEGylated lipid, dipalmitoylphosphatidylethanolamine (DPPE) bearing PEG 5,000 MW, for example, are also useful; about 8 mole percent DPPE is preferred. A preferred product which is highly useful for entrapping gas precursors contains 83 mole percent DPPC, 8 mole percent DPPE-PEG 5,000 MW and 5 mole percent dipalmitoylphosphatidic acid.

If desired, either anionic or cationic lipids may be used to bind anionic or cationic pharmaceuticals. Cationic lipids, such as those disclosed in U.S. Patent 5,283,185, which is incorporated herein by reference, may be used to bind DNA and RNA analogues within or on the surface of the gas or gas precursor-filled microbubble. A variety of other lipids such as DOTMA, DOTAP, and DOTB, are known to be useful. Lipids bearing cationic polymers such as polylysine or polyarginine may also be used to construct the microbubbles and enable binding of a negatively charged therapeutic, such as genetic material, to the outside of the microbubble. Additionally, negatively charged lipids may be used, for example, to bind positively charged therapeutic compounds. Phosphatidic acid, a negatively charged lipid, can also be used to complex DNA. Also, 5 to 10 mole percent phosphatidic acid in the liposomes improves the stability and size distribution of gas or gas precursor-filled liposomes.

Other useful lipids or combinations thereof apparent to those skilled in the art which are in keeping with the spirit of this invention are also encompassed by this invention. For example, carbohydrate-bearing lipids may be employed for *in vivo* targeting, as described in U.S. Pat. No. 4,310,505, the disclosure of which is hereby incorporated by reference. The most preferred lipids are phospholipids, preferably DPPC and DSPC, and most preferably DPPC.

Cationic polymers may be bound to the lipid layer through one or more alkyl groups or sterol groups which serve to anchor the cationic polymer into the lipid layer surrounding the gaseous precursor. Cationic polymers that may be used in this manner include, but are not limited to, polylysine and polyarginine, and their analogs such as polyhomoarginine or polyhomolysine. The positively charged groups of cationic lipids and cationic polymers, or perfluoroalkylated groups bearing cationic groups, for example, may be used to complex negatively charged molecules such as nucleic acids, thus binding the material to the surface of the microbubble. Alternatively, charged or uncharged molecules may be bound directly to the head groups of the lipids via ester, amide, ether, disulfide or thioether linkages.

Because gas-filled microbubbles are more stable than simple air or gas bubbles, they may be deployed as is to provide useful contrast enhancement; for example, if less than about four microns in diameter, they pass through the pulmonary circulation following peripheral venous injection, even when not specifically stabilized by one or more coating or emulsifying agents. One or more coating or stabilizing agents is preferred however, as are flexible stabilizing materials. Gas-filled microbubbles stabilized by polysaccharides, gangliosides, and polymers are more effective than those stabilized by albumin and other proteins. Liposomes prepared using aliphatic compounds are preferred for imaging applications, as gas-filled microspheres stabilized with these compounds are much more flexible and stable to pressure changes. Liposomes for drug delivery applications are preferably less stable, to facilitate microbubble breakage and drug release, and as a rule will incorporate less effective stabilizers, if any.

At least in part, the gas impermeability of gas-filled microbubbles has been found to be positively correlated with the gel state to liquid crystalline state phase transition temperature of the component lipids. For imaging applications, microbubbles exhibiting greater impermeability will be preferred and appropriate lipids can be chosen accordingly. See, for example, D. Marsh, *CRC Handbook of Lipid Bilayers*, CRC Press, Boca Raton, Fla. 1990, p. 139 for main chain melting transitions of saturated diacyl-sn-glycero-3-phosphocholines. However, it will require less energy to release a therapeutic compound from a microbubble with a lower gel to liquid crystal phase transition temperature, and targeted drug delivery applications will require the practitioner to select a balance between gas impermeability (and stability) and effective drug release.

In imaging applications, lipids having a phase transition temperature greater than about 37° C. are preferred for administration to humans, although microspheres having a gel to liquid phase transition temperature greater than about 20°C are adequate.

When the gas-filled microbubbles of this invention are liposomes, they are

preferably stabilized with lipids covalently linked to polymers of polyethylene glycol, commonly referred to as PEGylated lipids. Liposomes prepared from dipalmitoyl phosphatidylcholine are preferred for drug delivery applications, for their propensity to rupture on application of resonant frequency ultrasound or microwave radiation, while exhibiting adequate stability beforehand.

The microbubbles of the invention are preferably rendered stable to vascular recirculation. The microbubbles may be coated so to minimize uptake by the reticuloendothelial system. Useful coatings include, for example, gangliosides, glucuronide, galacturonate, guluronate, polyethyleneglycol, polypropylene glycol, polyvinylpyrrolidone, polyvinylalcohol, dextran, starch, phosphorylated and sulfonated mono, di, tri, oligo and polysaccharides and albumin. The liposomes may also be coated so as to evade recognition by the immune system.

Provided that the circulation half-life of the liposomes is sufficiently long, the antibody-conjugated microbubbles will adhere to and accumulate at endothelial sites expressing ICAM-1. Then, by focusing resonant sound waves on the selected tissue to be treated, any associated therapeutic will be released locally in the target tissue. For imaging applications, the antibody-conjugated microbubbles will provide a high-contrast site for ultrasound imaging, thereby revealing the existence, size, and location of damaged and/or activated endothelial cells.

For storage prior to use, the microbubbles of this invention may be suspended in an aqueous solution, such as a saline solution (for example, a phosphate buffered saline solution), or simply water, and stored preferably at a temperature of between about 2°C and about 10°C. The temperature of storage is preferably below the gel to liquid crystal phase transition temperature of the material forming the microbubble.

Bacteriostatic agents may also be included with the liposomes to prevent bacterial degradation on storage. Suitable bacteriostatic agents include but are not limited to benzalkonium chloride, benzethonium chloride, benzoic acid, benzyl alcohol, butylparaben, cetylpyridinium chloride, chlorobutanol, chlorocresol, methylparaben, phenol, potassium benzoate, potassium sorbate, sodium benzoate and

sorbic acid.

Various biocompatible gases may be employed in the gas-filled microbubbles of this invention, and gases suitable for liposome or microbubble encapsulation are known to the art. Such gases include air, nitrogen, carbon dioxide, oxygen, argon, xenon, neon, helium, sulfur hexafluoride, C1 to C6 fluorocarbons, or any and all combinations thereof. Gas precursors may be co-entrapped with other gases. For example, during the transition from the gas precursor to a gas in an enclosed environment containing an ambient gas, the two gases may mix and upon agitation and formation of microbubbles, the gaseous content of the microspheres will be a mixture of two or more gases.

The size of the microbubbles of this invention will depend upon the intended use. With the smaller liposomes, resonant frequency ultrasound will generally be higher than for the larger liposomes. Sizing also serves to modulate resultant liposomal biodistribution and clearance. The size of the liposomes can be adjusted, if desired, by procedures known to one skilled in the art, such as filtration, extrusion, sonication, homogenization, or the use of a laminar stream of a core of liquid introduced into an immiscible sheath of liquid. See, for example, U.S. Patent 4,728,578; U.K. Patent Application GB 2193095 A; U.S. Patent 4,728,575; U.S. Patent 4,737,323; International Application PCT/US85/01161; Mayer *et al.*, *Biochimica et Biophysica Acta* 1986, **858**, 161-168; Hope *et al.*, *Biochimica et Biophysica Acta* 1985, **812**, 55-65; U.S. Patent 4,533,254; Mayhew *et al.*, *Methods in Enzymology* 1987, **149**, 64-77; Mayhew *et al.*, *Biochimica et Biophysica Acta* 1984, **755**, 169-74; Cheng *et al*, *Investigative Radiology* 1987, **22**, 47-55; PCT/US89/05040; U.S. Patent 4,162,282; U.S. Patent 4,310,505; U.S. Patent 4,921,706; and *Liposomes Technology*, Gregoriadis, G., ed., Vol. I, pp. 29-37, 51-67 and 79-108 (CRC Press Inc, Boca Raton, Fla., 1984) Extrusion under pressure through pores of defined size is a preferred method of adjusting the size of the liposomes.

The preferred size range for the microbubbles of this invention, for purposes of cardiac vascular imaging or drug delivery, is a mean outside diameter of between

about 10 nanometers and about 20 microns, more preferably between about 30 nanometers and about 10 microns, most preferably from about one micron to about 5 microns. To provide therapeutic delivery to organs such as the liver, smaller microbubbles, between about 30 nanometers and about 100 nanometers in mean outside diameter, are preferred. For delivery to a tissue such as the kidney or the lung, the microbubbles are preferably less than about 200 microns in mean outside diameter.

The preferred route of administration is intravascularly. For intravascular use, the microbubbles are generally injected intravenously, but they may be injected intraarterially as well. The microbubbles of the invention may also be injected subcutaneously, into the lymphatic system, interstitially, or into any body cavity, as appropriate to the tissue being targeted. The microbubbles may be nebulized and inhaled for delivery to the alveoli.

Since certain gas filled liposomes may burst upon aggregation with lipids lining the targeted tissue, a therapeutic agent may be released after administration without the use of ultrasound. Thus, ultrasound irradiation will not in all cases be required to trigger drug release.

The useful dosage of targeted gas-filled microbubbles to be administered will vary depending on whether a therapeutic or diagnostic application is intended. In therapeutic applications, the dose will vary in the usual fashion with the weight and condition of the patient and the potency and efficacy of the therapeutic.

For use in ultrasonic imaging, the liposomes to be used with this invention preferably possess a reflectivity of greater than 2 dB, more preferably between about 4 dB and about 20 dB. Within these ranges, the highest reflectivity for the liposomes of the invention is exhibited by the larger liposomes, by higher concentrations of liposomes, and when higher ultrasound frequencies are employed.

For therapeutic drug delivery, the rupturing of the therapeutic containing microbubbles of the invention is carried out by applying ultrasound, preferably at the peak resonant frequency of the microbubbles, to the region of the patient where

therapy is desired, after the liposomes have been administered and have been allowed to concentrate at the activated endothelial cells in that region. The combination of targeted binding via the anti-ICAM antibody and localized irradiation provides a site-specific delivery not generally obtainable when either method of targeted delivery is used alone.

Preferably, the liposomes of the invention have a peak resonant frequency of between about 0.5 MHz and about 10 MHz. The peak resonant frequency of the gas-filled microbubbles of the invention will vary depending on the diameter and, to some extent, the elasticity or flexibility of the liposomes, but is readily determined empirically. Off-resonant radiation will also be effective, although less efficient, and will be accompanied by tissue heating.

For diagnostic or therapeutic ultrasound, any of the various types of diagnostic ultrasound imaging devices may be employed in the practice of the invention, the particular type or model of the device not being critical to the method of the invention. Also suitable are devices designed for administering ultrasonic hyperthermia, such devices being described in U.S. Patents 4,620,546, 4,658,828, and 4,586,512. Preferably, the device employs a resonant frequency (RF) spectral analyzer. The transducer probes may be applied externally or may be implanted. Ultrasound is generally initiated at lower intensity and duration, and then intensity and duration are increased, and the transducer is tuned to the resonant frequency, until the microbubbles are visualized (for diagnostic applications) or ruptured (for therapeutic applications).

By operating a diagnostic transducer in Doppler mode, power outputs up to 2.5 watts per cm^2 can be obtained. With the machine operating in Doppler mode, power can be delivered to a selected focal zone within the target tissue and the gas filled microbubbles can be made to release their therapeutics. For larger diameter microbubbles, e.g., greater than 3 microns in mean outside diameter, a lower frequency transducer may be more effective in accomplishing therapeutic release. For example, a lower frequency transducer of 3.5 megahertz (20 mm curved array model)

may be selected to correspond to the resonant frequency of the microbubbles. Using this transducer, 101.6 milliwatts per cm^2 may be delivered to the focal spot, and in Doppler mode the power output can be raised to 1.02 watts per cm^2.

To use the phenomenon of cavitation to release and/or activate the therapeutic within the microbubbles, lower frequency energies may be used, as cavitation occurs more effectively at lower frequencies. Using a 0.757 megahertz transducer driven with higher voltages (up to 300 volts), cavitation of solutions of will occur at thresholds of about 5.2 atmospheres.

Table 1 shows the ranges of energies transmitted to tissues from diagnostic ultrasound on commonly used instruments such as the PORTASCAN™ (Piconics Inc., Tyngsboro, Mass.) general purpose scanner with receiver pulser; the ECHOVIEW™ 8L Scanner including 80C System (Picker Inc., Cleveland, Ohio); or the Model D-9 VERSATONE™ (Medisonics Inc., Mountain View, Calif.) Bidirectional Doppler. In general, these ranges of energies employed in pulse repetition are useful for imaging the gas-filled microbubbles but are insufficient to rupture the microbubbles of this invention.

TABLE 1

Power and Intensities Produced by Diagnostic Equipment*

Pulse repetition rate (Hz)	Total ultrasonic power output P (mW)	Average Intensity at transducer face I_{TD} (W/m^2)
520	4.2	32
676	9.4	71
806	6.8	24
1000	14.4	51
1538	2.4	8.5

*Carson *et al.*, *Ultrasound in Med. & Biol.*, 3, 341-350 (1978).

In drug delivery applications, the sound energy may be pulsed, but continuous wave ultrasound is preferred in order to deliver higher energy levels. If pulsing is employed, the sound will preferably be pulsed in echo train lengths of at least about 8 and preferably at least about 20 pulses at a time. Machines designed for therapeutic ultrasound applications are preferred.

Either fixed frequency or modulated frequency ultrasound may be used. A modulated frequency is one in which the wave frequency changes over time, for example, from high to low (PRICH) or from low to high (CHIRP). For example, a PRICH pulse with an initial frequency of 10 MHz of sonic energy is swept to 1 MHz with increasing power from 1 to 5 watts. Focused, frequency modulated, high energy ultrasound may increase the rate of local gaseous expansion within the microbubbles and accelerate rupturing.

The frequency of the sound used may vary from about 0.025 to about 100 megahertz. Frequency ranges between about 0.75 and about 3 megahertz are preferred and frequencies between about 1 and about 2 megahertz are most preferred. Commonly used therapeutic frequencies of about 0.75 to about 1.5 megahertz may be used. Commonly used diagnostic frequencies of about 3 to about 7.5 megahertz may also be used. For very small microbubbles, e.g., below 0.5 micron in mean outside diameter, higher frequencies of sound may be preferred as these smaller microbubbles will absorb sonic energy more effectively at higher frequencies of sound. When very high frequencies are used, e.g., over 10 megahertz, the sonic energy will generally have limited depth penetration into fluids and tissues. External application may be preferred for the skin and other superficial tissues, but for deep structures, the internal application of sonic energy, for example, via interstitial or esophageal probes or intravascular ultrasound catheters may be preferred.

Where the targeted microbubbles are used for therapeutic delivery, the therapeutic compound to be delivered may be embedded within the wall of the microbubble, encapsulated in the microbubble, and/or attached to the microbubble, as desired. The phrases "attached to" or "incorporated in", or variations thereof, as used

herein in connection with the location of the therapeutic compound, means that the therapeutic compound is physically associated in some manner with the inside and/or the outside wall of the microbubble, such as through a covalent or ionic bond, hydrophobic interaction, or other means of chemical or electrostatic linkage or interaction. The phrase "encapsulated in" denotes that the therapeutic compound is located in the internal microbubble void. The phrase "embedded within" signifies the positioning of the therapeutic compound within the thickness of the microbubble wall itself. The phrase "comprising a therapeutic" denotes all of the varying types of therapeutic positioning in connection with the microbubble. Thus, the therapeutic can be positioned variably, such as, for example, entrapped within the internal void of the microbubble, situated between the gaseous interior and the internal wall of the microbubble, incorporated onto the external surface of the microbubble and/or enmeshed within the microbubble structure itself.

Any of a wide variety of therapeutics may be encapsulated in the microbubbles. By therapeutic, as used herein, it is meant an agent having beneficial effect on the patient. As used herein, the term therapeutic is synonymous with the terms contrast agent, nucleic acid, and drug. Genetic and bioactive materials may be incorporated into the internal gas-filled space of these microbubbles during the microbubble formation process or into or onto the lipid membranes of these particles. Incorporation onto the surface of these particles is preferred. Genetic materials and bioactive products with a high octanol/water partition coefficient may be incorporated directly into the lipid layer surrounding the gas but incorporation onto the surface of the gas-filled lipid spheres is more preferred. To accomplish this, groups capable of binding genetic materials or bioactive materials are generally incorporated into the lipid layers which will then bind these materials. In the case of genetic materials (DNA, RNA, both single stranded and double stranded and antisense and sense oligonucleotides) this is readily accomplished through the use of cationic lipids or cationic polymers which may be incorporated into the dried lipid starting materials.

Gas-filled microbubbles, when produced with phosphatidic acid, e.g.

dipalmitoylphosphatidic acid in molar amounts in excess of 5 mole % and preferably about 10 mole %, function as highly effective binders of genetic material. Such liposomes bind DNA avidly; see U.S. Patent 5,469,854. Compositions incorporating phosphatidic acid are more robust for diagnostic ultrasound and are useful for carrying DNA as well as other pharmaceuticals.

The ICAM-targeted microbubbles of the invention can be used to deliver oligonucleic acids, antisense therapeutics, or drugs such as cardiac glycosides, angiogenic factors and vasoactive compounds to ischemic regions of the myocardium, and to regions not yet ischemic but exhibiting activated or damaged endothelial cells.

Preferred therapeutics include genetic material such as, for example, genes carried on expression vectors such as plasmids, phagemids, cosmids, yeast artificial chromosomes (YACs), and defective or "helper" viruses, antisense nucleic acids, both single and double stranded RNA and DNA and analogs thereof, such as peptide nucleic acids and phosphorothioate, phosphoroamidate, and phosphorodithioate oligodeoxynucleotides. Additionally, the genetic material may be combined, for example, with proteins or other polymers.

In addition to known therapeutics, the microbubbles of this invention enable the targeted delivery of prodrugs, in particular prodrugs that are rapidly cleaved upon exposure to serum enzymes. When a gas-filled microbubble containing such a prodrug is burst, *e.g.* by ultrasound irradiation, the prodrug encapsulated by the liposome will then be exposed to the serum.

By way of example, a drug may be acylated at a hydroxyl group, providing an ester linkage which would readily be cleaved *in vivo* by enzymatic action in serum. The acylated prodrug is encapsulated within the antibody-conjugated gas-filled microbubble of the invention. When the microbubble is burst, for example by applied ultrasound, the prodrug encapsulated by the microbubble will then be exposed to the serum. The ester linkage is then cleaved by esterases in the serum, thereby generating the drug *in situ*, whereby the targeting action of the anti-ICAM antibody will have concentrated the released drug at an ICAM-expressing site in the body.

Ultrasound may be utilized not only to rupture gas-filled microbubbles, but also to thermally raise the microbubble walls to the phase transition temperature, thereby increasing the rate of chemical cleavage or physical release of the active drug from the microbubble.

The chemical structure of the therapeutic may be selected or modified to achieve an appropriate solubility such that the therapeutic may either be encapsulated within the internal gas-filled space of the microbubble, attached to the microbubble, or inserted wholly or in part into in the microbubble wall. A surface-bound therapeutic may for example bear one or more fatty acyl chains which serve to anchor the therapeutic to the microbubble wall; the acylated therapeutic will ideally be susceptible to acylase enzymes or will have inherent therapeutic properties. Alternatively, the therapeutic may be conjugated to a hydrophobic group having an aromatic or sterol structure, which can be incorporated into the surface of the microbubble and serve to anchor the therapeutic. By way of example, a therapeutic may be attached to the 3-hydroxyl group of cholesterol.

Methods for conjugating proteins such as antibodies to other proteins, to small molecules, and to surfaces, are well-known in the art. However, the conjugation of antibodies or other proteins with gas-filled microspheres, in a manner that permits selective binding of the microspheres to antigens on cell surfaces, had not been achieved prior to this invention. Various attempts to achieve this goal have been reported; see for example Torchlin *et al., Biochem. Biophys. Res. Commun.* 1978, **85**, 983-990; Endoh *et al., J. Immunol. Methods*, 1981, **44**, 79-85,; *Hashimoto et al., J. Immunol. Methods*, 1983, **62**, 155-162; Martin *et al., Biochemistry*, 1981, **20**, 4229-4238, Demos *et al., J. Pharm. Sci.*, 1997, **86**, 167-171, and Lanza and Wickline, WO 96/41647.

The embodiments of the invention which are described below demonstrate that microbubbles conjugated to a ligand for a specific molecular epitope selectively adhere to a biological surface expressing this epitope. They show that a novel perfluorocarbon gas-filled microbubble of the present invention, incorporating

monoclonal antibody to human ICAM-1 on its outer shell, preferentially binds to activated human coronary artery ECs that over-express ICAM-1. This appears to be the first demonstration of targeted microbubble attachment to cells expressing a surface protein seen in early atherosclerosis. These data demonstrate the principle of *in vivo* ultrasound imaging to identify cell markers which are pathognomonic of disease states and which are not otherwise identifiable *ante-mortem*.

The examples described herein demonstrate that a 40-fold increase in the extent of bubble adhesion occurred when activated ECs were exposed to anti-ICAM-1-conjugated microbubbles. The lack of adherence of plain microbubbles to normal or activated endothelium indicates that the components of the lipid shell have no avidity for ECs. This observation, and the fact that non-specific IgG microbubbles also do not adhere, indicate that anti-ICAM-1-conjugated bubble adhesion to activated cells is due to a specific interaction with ICAM-1. This interaction is further supported by flow cytometry data confirming up-regulation of ICAM-1 in IL-1β-exposed ECs. Interestingly, there is limited binding of anti-ICAM-1-conjugated microbubbles to ECs under basal conditions, presumably because of low level constitutive expression of ICAM-1 by normal ECs.

The data suggest that although adherence may decrease at the higher wall shear rates (1000 sec^{-1}) present in the microvasculature, the number of targeted microbubbles binding to activated ECs is still greater than that binding to normal cells perfused at this shear rate. Thus, despite the high wall shear rates present at the microvascular level, differential adherence of targeted bubbles to activated ECs can occur. Importantly, at the wall shear rate of 100 sec^{-1}, which characterizes larger arteries, a substantial number of targeted bubbles continue to adhere to activated ECs.

The presently ultrasound imaging agents of this invention, which are specific to a molecular marker of early endothelial disease, are expected to be useful for the identification of incipient atherosclerosis. Tissue-specific ultrasound contrast imaging agents are also expected to have applications to other disease states. The incorporation of ICAM-binding ligands on microbubbles is expected to enable

ultrasound localization or characterization of components of atherosclerotic plaque and re-stenotic lesions, and should have other non-cardiac applications in fields such as oncology and transplant vasculopathy, or other disease states where ICAM expression is up-regulated in the affected tissues.

The disclosures of all of the patents, publications and patent applications cited, herein are incorporated by reference herein.

This invention is further described in the following examples, which illustrate the preparation and testing of the gas or gas precursor filled microbubbles. The examples are provided by way of illustration only, and are not intended to limit the scope of the claims in any way.

EXAMPLES

Example 1a. Gas precursor filled microbubbles, preparation method #1.

The anti-ICAM-1 antibodies of this invention are commercially available (e.g. from Endogen, Inc. or Immunotech, Inc.). Albumin microbubbles are commercially available (ALBUNEX, Molecular Biosystems, Inc.). Perfluorocarbon gas-filled microbubbles were prepared with monoclonal antibody on the shell as the ligand for EC binding. Perfluorobutane (PCR Inc.) was dispersed by sonication (XL2020™ sonicator, Misonix Inc.) in aqueous medium containing phosphatidylcholine (Avanti Polar Lipids Inc.), a surfactant, a phospholipid derivative containing a carboxyl group, and a fluorescein derivative of phosphatidylethanolamine (Molecular Probes, Inc.) in an approximate molar ratio of 75:15:7:1. The perfluorobutane was encapsulated during sonication by a lipid shell carrying the fluorescent label. The carboxylic groups were exposed to the aqueous environment and used for covalent attachment of antibodies to the microbubbles as follows: First, unbound lipid dispersed in the aqueous phase was separated from the gas-filled microbubbles by flotation. Second, carboxylic groups on the microbubble shell were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma), and antibody was then covalently attached via its primary amino groups with the formation of amide bonds (5). Unbound antibody was thereafter removed by flotation. Three different microbubble

species were produced, containing either anti-human ICAM-1 IgG monoclonal antibody (Endogen), purified non-specific mouse IgG (Sigma), or nothing (plain), on the shell. Microbubble diameter ranged from 1 to 10 microns and concentration was 10^8 to 10^9 bubbles/ml.

Example 1b. Gas precursor filled microbubbles, preparation method #2.

Functionalized polyethylene glycol oligomers are commercially available from Shearwater Polymers, Inc., or may be prepared by established methods. For the preparation of heterobifunctional ethylene glycol oligomers, see for example R. Herrmann *et al.*, EP 410,280.

A heterobifunctional polyethylene glycol oligomer, of molecular weight from about 500 to about 5,000, bearing a terminal carboxylic acid and a terminal 3-(2-pyridyldithio)-propionamido group, is prepared from the corresponding ω-amino acid $H_2N(CH_2CH_2O)_nCH_2COOH$ (n = 10-100) by treatment with 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP). This material, designated PDP-PEG-COOH, is added to a mixture of 1-ethyl-3-(3'dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide in dimethylformamide. Albumin microbubbles, suspended in water, are added, and after 24 hrs the resulting PDP-PEG-conjugated microbubbles are isolated by filtration.

The PDP-PEG-conjugated microbubbles are suspended in a phosphate buffer at pH 5.5, and excess dithiothreitol (DTT) is added to a concentration of 25 mM. The suspension is gently agitated for 30 minutes under a nitrogen atmosphere, and the resulting thiol-PEG conjugated microbubbles isolated by filtration and stored refrigerated, under nitrogen, until used.

Anti-ICAM-1 antibody is acylated with 3-(2-pyridyldithio)propionyl groups, by treatment with SPDP, as detailed in Demos *et al., J. Pharm. Sci.,* 1997, **86**, 167-171, but at a molar ratio of 5:1 rather than 15:1, to provide PDP-antibody.

PDP-antibody and thiol-PEG-microbubbles are combined in a pH 5.5 buffer, under a nitrogen atmosphere, and gently agitated for 24 hrs, to provide after filtration antibody-conjugated microbubbles.

Substantially the same result may be obtained by treating the PDP-antibody with DTT, as described by Demos *et al.*, *supra*, and combining the resulting thiolated antibody with PDP-PEG-microbubbles.

Example 2. Gas-filled microbubbles prepared from lipids.

N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine (MPB-PE) is prepared by the published method (R. New, Characterization of Liposomes, in *Liposomes: A Practical Approach*; Oxford University Press, New York, 1990; p. 183.)

MPB-PE and dipalmitoylphasphatidyl choline are combined, with the MPB-PE being present in from 5 to 10% molar ratio, in 0.9% aqueous sodium chloride, at a total lipid concentration of 25 mg/ml. The suspension is agitated and filtered, as described in U.S. Patent 5,469,854, to generate gas-filled liposomes.

PDP-PEG-COOH, prepared as in Example 1b, is coupled to anti-ICAM-1 antibody as follows: PDP-PEG-COOH is added to a mixture of a water-soluble carbodiimide such as 1-ethyl-3-(3'dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide in dimethylformamide. Antibody, dissolved in water, is added, and after 24 hrs the resulting PDP-PEG-conjugated antibody is isolated by chromatography on SEPHADEX™ G-50, eluting with a pH 7.0 phosphate buffer. This material is combined with excess DTT in a pH 5.5 buffer, and after 30 minutes the thiol-PEG-conjugated antibody is isolated by chromatography on SEPHADEX™ G-50, eluting with a deoxygenated pH 7.0 phosphate buffer. The material is stored refrigerated under nitrogen until used.

Thiol-PEG-conjugated antibody is added to a suspension of the MPB-PE containing gas-filled liposomes, and the foamy mixture is vigorously shaken for 20 hr under a nitrogen atmosphere. The resulting antibody-conjugated gas-filled liposomes are isolated by low-pressure filtration.

The above processes may be carried out under an atmosphere of perfluoropropane or perfluorobutane, and the resulting liposomes stored under these gases, if microbubbles filled with these gases is desired.

Rhinovirus coat proteins, the beta-2 integrin subunit, alphaL/beta2 integrin (LFA-1), and alphaM/beta2 integrin (Mac-1) may also be coupled to microbubbles, by any of the above methods.

Example 3. Binding of antibody-conjugated microbubbles to cultured endothelial cells.

Exposure of endothelial cells (ECs) to microbubbles was performed by use of a parallel plate perfusion chamber (Villanueva *et al.*, *J. Am. Coll. Cardiol.* 1997, **30**, 689-693) which permits contact between the cells and small, concentrated volumes of microbubbles within a closed system containing culture medium. Briefly, coverslips (2.65 cm^2) with confluent monolayers of ECs were mounted in a 200 micron-high perfusion chamber with the ECs facing the interior of the chamber. A SILASTIC™ gasket and vacuum system were used to seal the compartment and secure the coverslip in place. Entry and exit ports on the chamber were used to prime the system with serum-free culture medium (Clonetics, Inc.). The entry port was fitted with a latex catheter cap which enables the introduction of microbubbles on to the endothelial surface via a 20G needle. The exit port was connected via SILASTIC tubing to a syringe pump (Harvard Apparatus, Inc.) set in the withdrawal mode, which permits perfusion of the chamber when the entry port is connected to a reservoir of perfusate. Wall shear rate during perfusions was regulated by adjusting the rate of withdrawal by the syringe pump. The chamber was mounted on the stage of an inverted epifluorescent microscope (Zeiss AXIOVERT 35™) connected to a videocamera.

Human coronary artery ECs (Clonetics Corp) were subcultured at 37°C on glass coverslips and grown to confluence over 4 days in serum-free culture medium comprised of endothelial basal media (Clonetics). To create inflammatory endothelium, interleukin-1β (IL1β) (Sigma) was added to the cultures 4 hours prior to the experiment.

Lipid-derived microbubbles containing perfluorobutane gas were prepared as described above. Microbubbles were coupled either to murine anti-human ICAM-1 monoclonal antibody (Endogen) or to non-specific murine immunoglobulin (IgG)

(Sigma). Microbubbles (size 2-3 microns) resulting from the coupling procedure were suspended in Hepes buffer at a concentration of 2 - 3 x 10^8 bubbles/ml. The total volume of each sample was 1-2 ml. Prior to the experiment, the samples were centrifuged at 1000 rpm for 5 minutes, the buffer was carefully removed from the foamy bubble layer, replaced with an equivalent volume of serum-free culture medium, and the centrifugation and resuspension steps were repeated a second time. Following the centrifugation and resuspension procedure, a sample was examined under fluorescent microscopy to qualitatively confirm microbubble integrity.

After each completion of the experiment, coverslips were lightly fixed in 2% paraformaldehyde, washed in PBS 3 times, incubated with 0.1% Triton-X in 2% paraformaldehyde for 5 minutes, followed by 3 additional washes with PBS. To stain for F-actin, the coverslips were incubated with rhodamine-conjugated phalloidin for 30 minutes, resuspended in 1 ml PBS, and washed 3 times with PBS. Phalloidin binds with high affinity to F-actin and can be used as a qualitative indicator of EC activation. Hoechst stain was used to identify the nuclei. The slides were mounted in GELVATOL™ and placed onto a coverslip for light microscopy (Nikon FXA™ microscope).

Flow cytometry was used quantify cell surface expression of intercellular adhesion molecule-1 (ICAM-1) (Villanueva *et al., J. Am. Coll. Cardiol.* 1997, 30, 689-693). Briefly, EC monolayers were harvested with collagenase (0.2mg/ml) and 2mM EDTA in PBS after incubation with or without IL for 4.5 hours. The harvested cells were washed with PBS and incubated for 20 minutes at 4°C, rewashed with phycoerythrin (PE) -conjugated antihuman ICAM-1 murine monoclonal IgG (3.12μg/ml, Immunotech) or nonspecific PE-conjugated murine IgG (5μg/ml, Becton Dickinson). The cells were washed another 2 times and subsequently fixed in 1% paraformaldehyde in PBS. Samples were analyzed for fluorescence (540 nm for PE) with a fluorescence activated cell sorter (FACSCAN™, Becton-Dickinson Inc.), and five thousand events were analyzed for each sample.

A coverslip with confluent ECs was mounted in the perfusion chamber and the

system was primed with serum-free culture medium and 10^{-5} M mepacrine (Sigma) to fluorescently label the ECs. A volume of 0.2ml of microbubbles, approximately equivalent to the total volume of the perfusion chamber, was drawn into a 1cc tuberculin syringe and slowly injected with a 20G needle through the latex catheter cap directly into the chamber, replacing the 0.2 ml volume of priming culture medium. The microbubbles were allowed to dwell with the ECs under static conditions. Because of microbubble buoyancy, the chamber was inverted, with the ECs forming the roof of the chamber, in order to maximize exposure of cells to bubbles. After a 3 minute dwell time, the chamber was perfused for 3 minutes with bubble-free culture medium to wash unbound bubbles from the system.

Experiments were separately performed under two different conditions: with normal endothelium or with IL-1β activated endothelium. Each coverslip was exposed to one of three preparations: microbubbles conjugated to anti-human ICAM-1 monoclonal Ab, microbubbles conjugated to non-specific IgG, or microbubbles without conjugated protein.

After each perfusion, 20 randomly selected microscopic fields (1000x) per coverslip were examined using epifluorescent videomicroscopy. The number of bubbles and endothelial cells per field were counted. Selected coverslips were thereafter fixed in paraformaldehyde and prepared for multicolor fluorescent microscopy as described above.

Comparisons with respect to numbers of adherent microbubbles per coverslip were made between and within experimental groups using t-testing. Statistical significance is defined as $p < 0.05$ (two-tailed).

RESULTS

Data from 40 coverslips perfused at a wall shear rate of 25 sec-1 are expressed in terms of total number of adherent bubbles normalized to the number of cells observed per coverslip, and are summarized in Figure 1. Binding of all microbubble types to normal ECs cells was minimal. There was a significant interaction between EC activation status and bubble type by ANOVA [F (2,34) for interaction=3.65]. In

post-hoc analysis, it was found that, as predicted, there was a greater number of anti-ICAM-1 labeled bubbles adhering to activated ECs (8.0±3.5) compared to normal ECs (0.21±0.09, $p<0.001$). In contrast, there was no difference in the number of "plain" microbubbles adhering to normal (0.04±0.02) vs. activated (0.05±0.02) ECs ($p=0.42$). Similarly, bubbles containing non-specific IgG had no preference for activated (0.04±0.02) vs. normal (0.03±0.01) endothelium.

Figures 2 shows fluorescent micrographs of ECs exposed to non-specific IgG-labeled bubbles (2A and 2B) or anti-ICAM-1-containing bubbles (2C and 2D) under normal conditions or after endothelial activation with IL-1β. EC nuclei appear blue, rhodamine-labeled F-actin filaments are red, and microbubbles exhibit green fluorescence. Figure 2 shows no adherence of non-specific IgG-conjugated bubbles to ECs under basal (Fig 2A) or activated (Fig 2B) conditions. There is scant adherence of anti-ICAM-1-containing microbubbles to ECs under basal conditions (Fig 2C), and extensive adherence of these bubbles to activated ECs (Fig 2D). There is dense rhodamine-staining of F-actin in the IL-1β-stimulated cells, indicative of activation. Fig 2E shows a single activated EC with multiple adherent anti-ICAM-1 microbubbles.

In the 3 coverslips with activated ECs exposed to anti-ICAM-1 microbubbles and perfused sequentially at 100 sec^{-1} and 1000 sec^{-1}, the number of adherent microbubbles decreased from 2.6 ± 0.3 to 0.8 ± 0.4, respectively ($p=0.04$). There was minimal adherence of the targeted microbubbles to normal ECs on the 2 coverslips perfused successively at 25 sec^{-1} (0.2 ± 0.0) and 1000 sec^{-1} (0.1 ± 0.0, $p=0.25$). Despite the relatively small number of coverslips tested, binding of anti-ICAM-1 bubbles appeared to occur preferentially to activated ECs (0.8 ± 0.4) as compared to unstimulated cells (0.1 ± 0.0) even at wall shear rates of 1000 sec^{-1} ($p=0.08$). The relative distribution for ICAM-1 binding (number of events vs. fluorescent intensity) in normal and IL-1β stimulated cells is shown in Figure 3, which indicates a shift in the extent of ICAM-1 expression following IL-1β stimulation. The percent of ECs expressing ICAM-1 above background (non-specific IgG binding) was increased

($p<0.0001$) after IL-1β exposure (83±5%, n=4 coverslips) compared to baseline (39±10%, n=5 coverslips).

CLAIMS

What is claimed is:

1. Gas-filled microbubbles conjugated to a molecule having binding affinity for ICAM-1.

2. The microbubbles of claim 1, wherein the molecule having binding affinity for ICAM-1 is an anti-ICAM-1 antibody.

3. An echogenic contrast agent preparation, comprising a suspension of the microbubbles of claim 1 or claim 2 in a pharmaceutically acceptable vehicle.

4. A method of imaging a tissue or a portion thereof, which tissue or portion thereof comprises ICAM-1 expressing cells, the method comprising the steps of:

(a) contacting said tissue with gas-filled microbubbles conjugated to molecule having binding affinity for ICAM-1,

(b) allowing said microbubbles to bind to said cells, and

(c) obtaining an ultrasound image of said tissue.

5. The method of claim 4, wherein said molecule having binding affinity for ICAM-1 is an anti-ICAM-1 antibody.

6. The method of claim 4 or claim 5 wherein said cells are activated coronary endothelial cells.

7. The method of claim 4 or claim 5, wherein said tissue is coronary vascular tissue.

8. The microbubbles of claim 1 or claim 2, wherein said molecule having binding affinity for ICAM-1 is conjugated to said microbubble by means of a poly(ethylene glycol) linker.

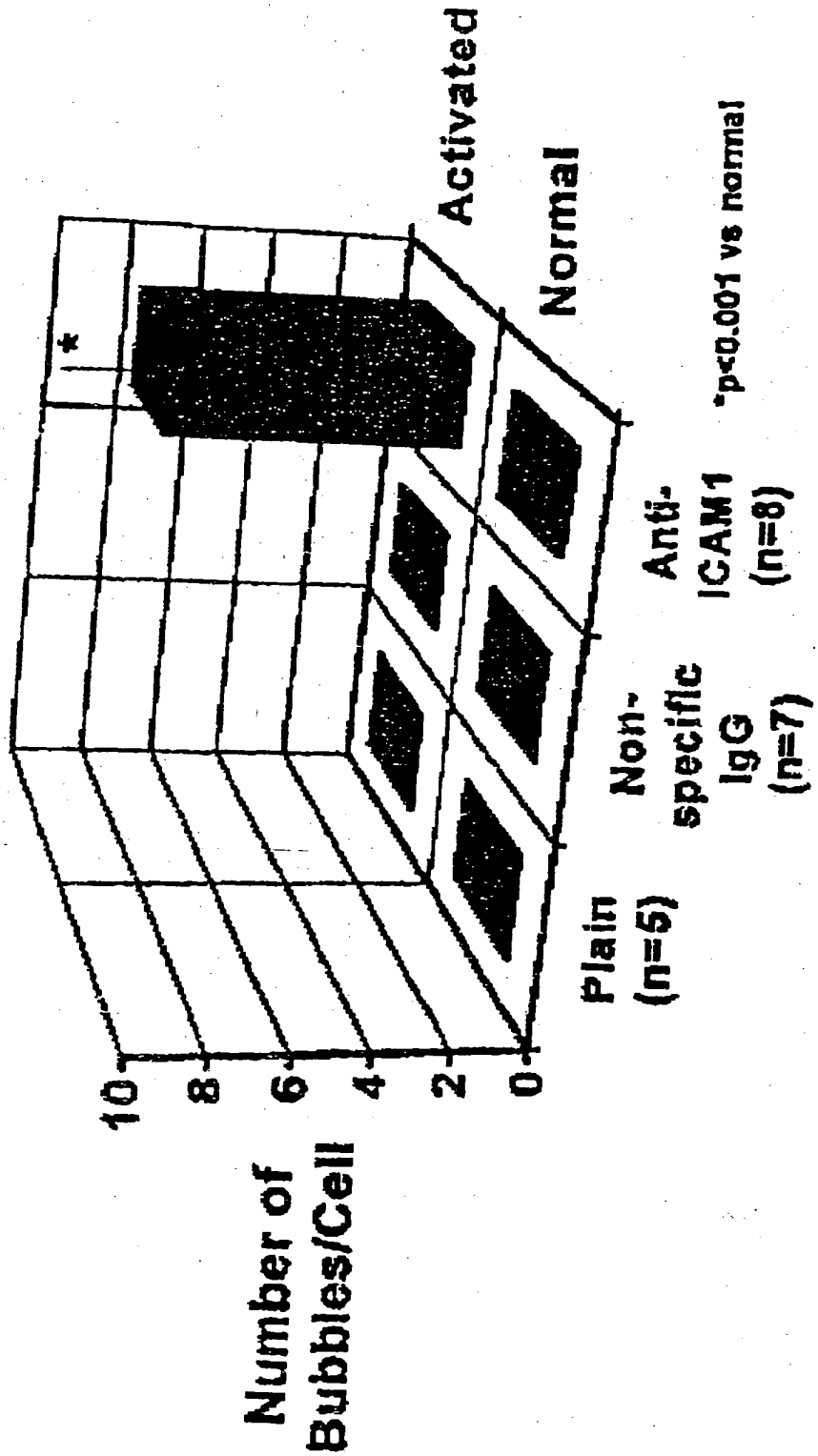
Activated
Normal
*
Number of Bubbles/Cell
10
8
6
4
2
0
Plain (n=5)
Non-specific IgG (n=7)
Anti-ICAM1 (n=8)
*p<0.001 vs normal

Figure 1

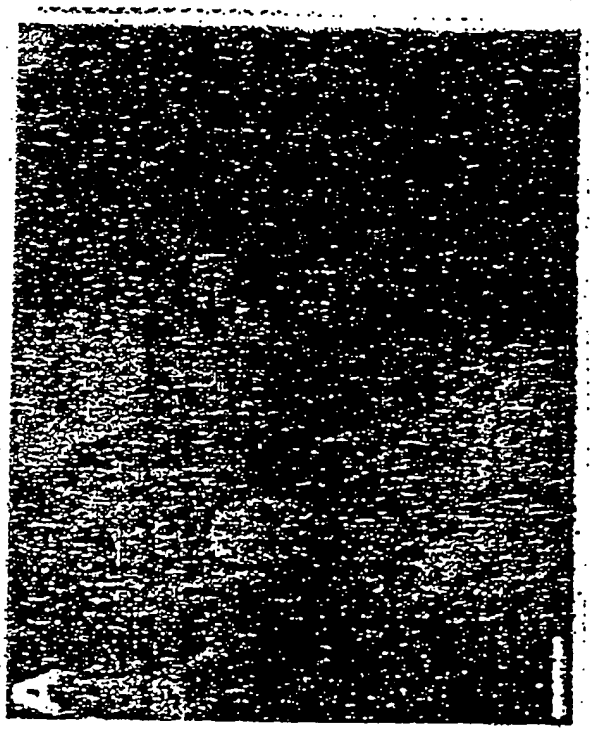

Figure 2A

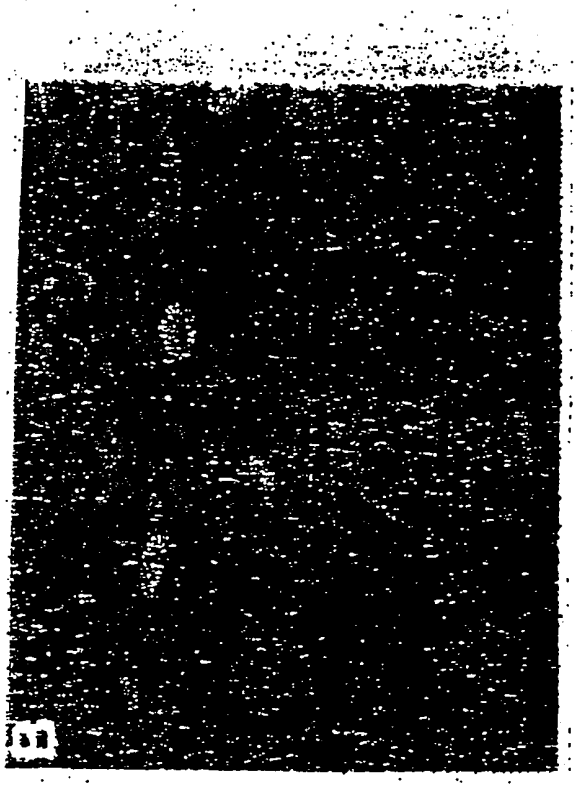

Figure 2B

Figure 2C

Figure 2D

Figure 2E

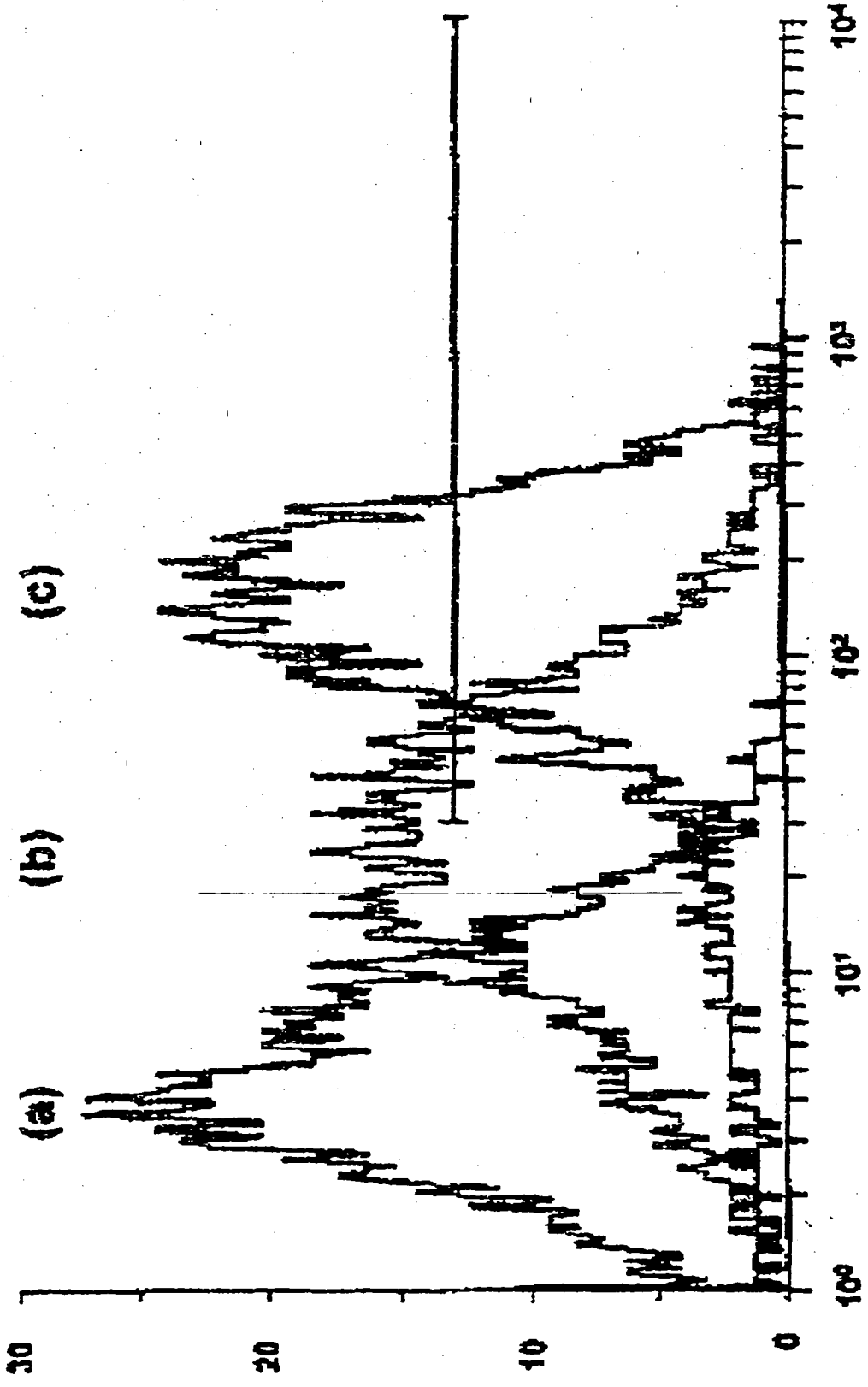

Figure 3